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Monoclonal antibodies that prevent adhesion of B 16 melanoma cells and reduce metastases in mice: Crossreaction with human tumor cells

(cancer immunotherapy/human melanoma/tumor-associated antigen/syngeneic immunization)

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ABSTRACT **Monoclonal antibodies raised against B 16 melanoma cells in syngeneic mice were functionally screened for their ability to inhibit cell adhesion in tissue culture. Three of these antibodies (16/43, 16/77, 16/82), when preinjected into C57BL/6 mice, markedly reduced the number of experimental lung metastases produced by B 16 cells, possibly by interference with their adhesion to the lung endothelia. We now report that these monoclonal antibodies block *in vitro* attachment of the majority of human melanoma cell lines tested and also of carcinoma, neuroblastoma, and glioblastoma cells from both mice and humans but untransformed cell lines such as 3T3 mouse or MRC-5 human fibroblasts are not affected. The antibodies also react with mouse teratocarcinoma stem cells (F9, PCC4) but not with differentiated teratocarcinoma lines (PYS-2, 944). Furthermore, the antiadhesion activity of the antibodies could be quantitatively absorbed by intact human and mouse tumor cells but not by untransformed cells, suggesting that the corresponding antigens may represent tumor-associated cell surface components. Correspondingly, the antigens were found on simian virus 40-transformed 3T3 mouse fibroblasts and are expressed in a temperature-sensitive fashion in chicken fibroblasts transformed with a temperature-sensitive Rous sarcoma virus. On "immunoblots" of NaDodSO$_4$-containing gels the three selected antibodies (16/43, 16/82, 19/1) were absorbed by antigens with molecular weights of 40,000 and 50,000.**

Monoclonal antibodies are highly specific reagents; in cancer research, they may eventually develop into powerful tools for diagnosis and therapy. However, few cases have been reported so far in which monoclonal antibodies were used with the goal of suppressing human tumors (1–4); most studies have dealt with human tumor cells in *nude* mice or with animal tumors (5–11). It is clear from these studies that the success of using monoclonal antibodies for cancer therapy ultimately depends on two factors: (*i*) whether the antibodies are specific for the tumor cells (i.e., are not absorbed by normal tissue) and (*ii*) whether the antibodies will also functionally interfere with tumor development (e.g., an antibody selected simply for binding to tumor cells might not be functionally active).

We have recently developed procedures to find antibodies that fulfill these conditions. First, monoclonal antibodies were produced against tumor cells by immunization of syngeneic animals; this procedure enriches for antibodies directed against the "mutant" characteristics of the tumor cell—i.e., against tumor-associated antigens. Second, these monoclonal antibodies were screened in functional assays *in vitro*; this procedure increases the chance of finding antibodies that interfere with tumor development *in vivo*. Thus, we found eight monoclonal antibodies against B 16 mouse melanoma that blocked adhesion of the melanoma cells in tissue culture (12). These antibodies were directed against antigens found on the surface of B 16 melanoma cells but less on untransformed mouse cells. The antigens were not detected on normal mouse tissues (e.g., lung, kidney, liver, spleen) but are expressed in lungs colonized by B 16 melanoma cells. Significantly, three of these antibodies markedly reduced the number of experimental lung metastases produced by highly invasive B 16 clones injected into the animals' bloodstream.

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MATERIALS AND METHODS

Animals and Cell Lines. The human melanoma cell lines were provided by J. P. Johnson (Munich) (13); Cloudman melanoma, mouse neuroblastoma (NB41A3), simian virus 40 (SV40)-transformed 3T3 fibroblasts, and human embryonic lung fibroblasts (MRC-5) were obtained from Flow Laboratories; mouse hepatoma cells were a gift of R. Kemler (Tübingen); human Wilm tumor cells (TuWi) and human lung carcinoma cells (A549) were obtained from R. Braun (Heidelberg); Tagli glioblastoma cells (human) were a gift of B. Odermatt (Zürich); CSG 120/7 (transformed mouse epithelial cell line) was provided by L. M. Franks (London) (14); and HT-29 (human colon carcinoma cells), TR126 (human tongue carcinoma cells), TR138 (human larynx carcinoma cells), and MCF7 (human breast carcinoma cells) were obtained from E. B. Lane (London) (15). Chicken embryo fibroblasts (CEFs) transformed with Rous sarcoma virus (RSV) (SR-1) and with a temperature-sensitive RSV (*ts*68) were a gift of H. Bauer (Giessen).

Attachment and Absorption Assays. The hybridoma supernatants were used directly for the attachment and adsorption assays as described (13). Alternatively, the antibodies were purified from ascites fluids on protein A-Sepharose (for IgG2a) or by precipitation at low ionic strength followed by chromatography on lens culinaris lectin-Sepharose (for IgM).

Determination of the Molecular Weights of the Antigens. Melanoma cells grown to confluency on 15-cm tissue culture dishes were scraped off with a rubber policeman and rapidly taken up in hot NaDodSO$_4$ dissociation buffer (16) containing 1 mM phenylmethylsulfonyl fluoride. Cellular protein (80 μg per lane, equivalent to 140,000 cells) was electrophoresed on 5–15% NaDodSO$_4$/polyacrylamide gradient gels and then blotted onto nitrocellulose filters according to Towbin *et al.* (17). Erythrocyte membranes served as molecular weight markers. The amido-black-stained filters were saturated with hemoglobin at 1 mg/ml and gelatine at 0.1 mg/ml for 30 min and then cut into 2-mm pieces. These were incubated individually with 100 μl of hybridoma supernatant at 4°C overnight, and residual

Abbreviations: SV40, simian virus 40; CEF, chicken embryo fibroblast; RSV, Rous sarcoma virus.

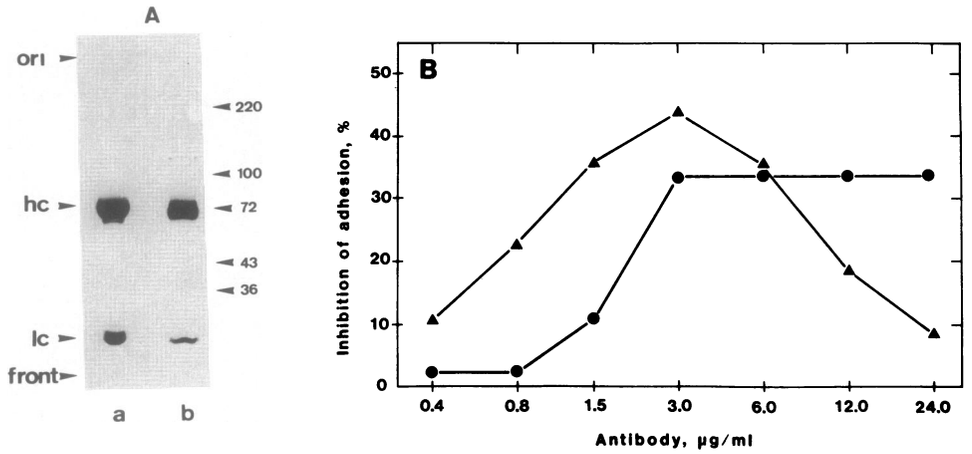

FIG. 1. Effect of purified monoclonal antibodies on cell adhesion in tissue culture. (*A*) $NaDodSO_4$ gel electrophoresis of antibody 16/82 (an IgM) as purified by precipitation at low ionic strength (lane a). An autoradiogram of a metabolically [^{35}S]methionine-labeled 16/82 hybridoma supernatant is shown in lane b. Numbers on the right represent $M_r \times 10^{-3}$. (*B*) Dependence of the antiadhesion activity with B 16 mouse melanoma cells on antibody concentration. ▲, Antibody 16/82; ●, antibody 16/77 (an IgG2a). ori, Origin; hc, heavy chain; lc, light chain.

Table 1. Reactivity of anti-B 16 monoclonal antibodies with human melanoma cell lines: Effect on cell adhesion *in vitro*

	Inhibition of adhesion, comparison with control									
Antibody	A375	SK-Mel-25	Strömer	MelJuSo	IGR3	ParL	MelWei	MelHo	MelIm	MelJu
16/51	++++	++++	++++	++++	++++	+++	++	+	+	−
16/76	++++	++++	++++	++++	+++	+++	++	+	−	−
19/1	++++	++++	+++	+++	++	−	+	−	+	−
16/81	++++	++++	+++	+++	++	+	−	−	−	−
16/43*	++++	++++	+++	+++	++	−	−	−	−	−
16/77*	++++	++++	+++	+++	++	+	−	−	−	−
16/82*	++++	++++	+++	++	++	+	−	−	−	−
16/56	++++	+++	+++	++	+	+	−	−	−	−

Inhibition of adhesion is represented as follows: ++++, 80–100%; +++, 60–80%; ++, 40–60%; +, 20–40%; −, 0–20%.
*These monoclonal antibodies prevented the formation of experimental lung metastases, when injected into C57BL/6 mice before injection of B 16 mouse melanoma cells (ref. 12).

activity in the supernatant was determined by the attachment assay described above.

RESULTS

Selected monoclonal antibodies against B 16 melanoma that prevented cell adhesion in tissue culture and reduced experimental metastases in mice (12) were purified from ascites fluids (Fig. 1*A*) and their concentration dependence was examined in the *in vitro* adhesion assay. The antibodies fell into two categories, one type that was equally active at μg/ml and higher concentrations (represented by antibodies 16/77 and 16/43, Fig. 1*B*) and a second type that was active only at μg/ml concentrations (represented by antibody 16/82, Fig. 1*B*). During purification, antiadhesion activity of IgG2a was retained on protein A-Sepharose and of IgM on lens lectin-Sepharose.

The Monoclonal Antibodies Crossreact with Human Melanoma Cells. These antibodies were tested for their ability to affect the adhesion of 10 human melanoma cell lines in tissue culture (Table 1). They strongly crossreacted with most of the human lines; the cells fitted into three categories, those strongly inhibited by the antibodies (A375, SK-Mel-25, Strömer, and JuSo), those inhibited to an intermediate degree (IGR3 and ParL), and those weakly inhibited or not blocked (MelWei, MelHo, MelIm, and MelJu).

Subclones of selected human melanoma lines were also examined in the *in vitro* adhesion assay (Table 2). Clearly, heterogeneities could be observed.

The Anti-B 16 Monoclonal Antibodies Crossreact with Other Types of Tumor Cells but not with Nontransformed Cell Lines. A series of mouse tumor cell lines was tested for crossreaction with the described anti-B 16 monoclonal antibodies (Table 3). The antibodies did not significantly interfere with *in vitro* adhesion of Cloudman mouse melanoma cells (except for antibody 16/51) but crossreacted with CSG 120/7 mouse carcinoma cells, and most of the antibodies also reduced the adhe-

Table 2. Reactivity of anti-B 16 monoclonal antibodies with human melanoma cell lines: Heterogeneity of subclones of MelHo

Anti-	Subclone, inhibition of adhesion									
body	1	2	3	4	5	6	7	8	9	10
16/51	++	+	−	+	+++	+++	+	−	+++	++
16/82	−	−	−	−	−	−	−	−	−	−

Subclones were prepared as described (12). Inhibition of adhesion is represented as in Table 1.

Table 3. Reactivity of anti-B 16 monoclonal antibodies with mouse cell lines: Effect on adhesion *in vitro*

	Inhibition of adhesion, comparison with control					
Antibody	B 16-F 1 melanoma	Cloudman melanoma	CSG 120/7 carcinoma*	Hepatoma	Neuroblastoma	3T3
16/51	+++	++	+++	+++	+++	−
16/76	+++	−	+	−	−	−
19/1	++	−	+	−	+	−
16/81	+++	−	+	++	++	−
16/43	++	−	++	++	++	−
16/77	++++	−	+	+	+	−
16/82	++	−	+	+	−	−
16/56	+++	−	+	−	−	−

Inhibition of adhesion is represented as in Table 1. Control cells were plated in regular medium.
*CSG 120/7 is a chemically transformed epithelial cell line from mouse salivary gland that produces carcinoma-like tumors.

Table 4. Reactivity of anti-B 16 monoclonal antibodies with other human cell lines: Effect on *in vitro* adhesion

Antibody	Inhibition of adhesion, comparison with control									
	HeLa	TuWi	A549	A431	MCF7	HT-29	TR126	TR138	Tagli	MRC-5
16/51	+++	++++	++++	+++	+++	++++	+++	++++	++++	−
19/1	++	++	+++	++	++	+++	+	++++	++	−
16/81	++++	++	+++	++	++	++	++	++	++	−
16/43	++	++	+++	++	+++	++++	++++	++++	++	−
16/77	+++	+	+++	++	++	++	++	++++	+++	−
16/82	+	+	+	+	++	+	++	++++	++	−

Inhibition of adhesion is represented as in Table 1. HeLa, TuWi, A549, A431, MCF7, and HT-29 are human tumor cell lines established for some years; TR126, TR138, and Tagli are recently derived lines. MRC-5 is a "normal" human embryonic lung fibroblast; TuWi, Wilm tumor; A549, lung carcinoma; A431, vulva carcinoma; MCF7, breast carcinoma; HT-29, colon carcinoma; TR126, tongue carcinoma; TR138, larynx carcinoma.

sion of mouse hepatoma and mouse neuroblastoma cells. No effect on 3T3 fibroblasts was seen. Similarly, a series of other human tumor cells was screened with the antibodies (Table 4).

Table 5. Reactivity of anti-B 16 monoclonal antibodies with transformed and untransformed cell lines

Anti-body	Inhibition of adhesion, comparison with control					
			5th passage		12th passage	
	3T3	SV40-3T3	CEF	RSV-CEF	CEF	RSV-CEF
19/1	−	−	++	++	−	++
16/81	−	+	++	++	−	+
16/43	−	+	++	++	+	+++
16/77	−	+	++	++	−	++
16/82	−	+	+++	++	+	+++
16/56	−	++				
16/76	−	++				

Inhibition of adhesion is represented as follows: +++, 75–100%; ++, 50–75%; +, 25–50%; −, 0–25%. RSV-CEF, RSV-transformed CEFs.

Again, extensive crossreaction with various carcinoma cells and a glioblastoma cell line was observed. The untransformed human cell line MRC-5 (embryonal lung fibroblasts) was negative in the assay.

In further adhesion experiments, virally transformed mouse and chicken fibroblasts were examined (Table 5). In contrast to 3T3 cells, the adhesion of SV40-transformed 3T3 fibroblasts was inhibited by the antibodies. CEFs were also prevented from adhesion at early passages (e.g., 5th passage) but were virtually unaffected later (e.g., 12th passage). Transformation with RSV maintained adhesion sensitivity at this later stage (Table 5). Similarly, the antibodies were significantly more effective with CEFs transformed by a temperature-sensitive RSV (*ts68*) at the permissive than at the nonpermissive temperature (Table 6).

Biochemical Characterization of the Antigens. We carried out a series of antibody absorption experiments with living cells and subsequently tested the supernatants for residual activity. As examples (Fig. 2 *A* and *B*), antibodies 16/82 and 19/1 were absorbed by 5×10^4 human IGR3 and A375 melanoma cells (strongly reacting in the adhesion assay) but much less by MelHo

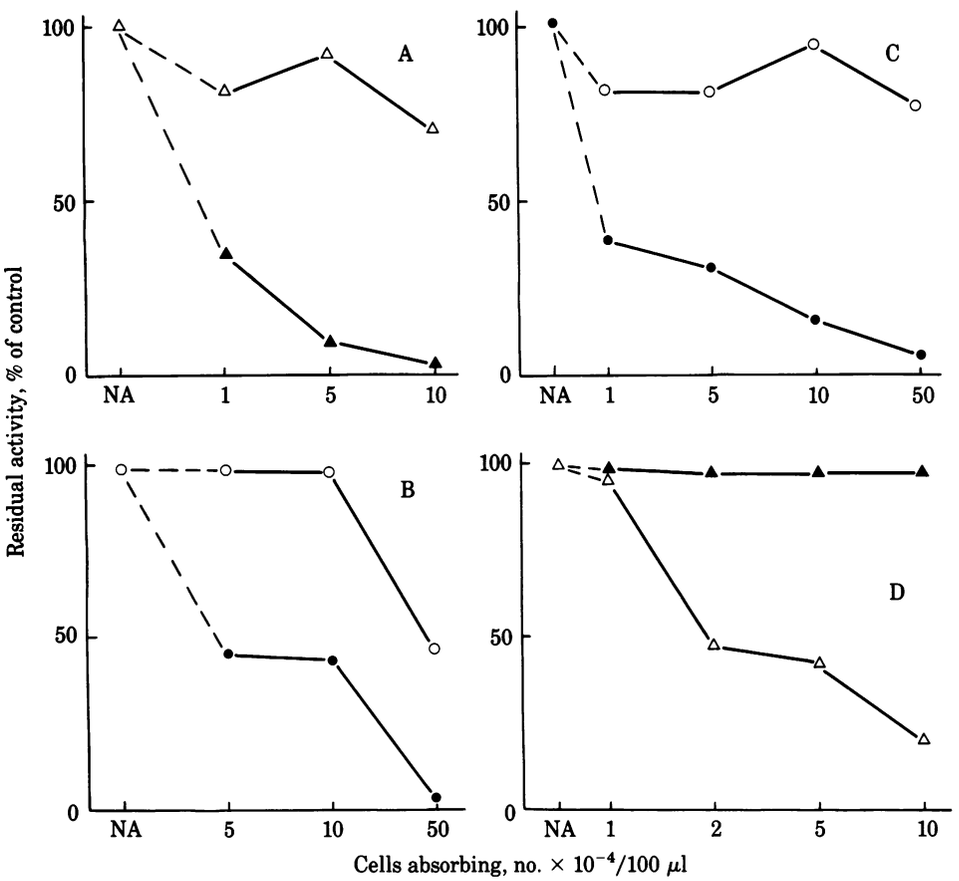

FIG. 2. Absorption of monoclonal antibodies 16/82 (*A* and *C*) and 19/1 (*B* and *D*) by intact tumor cells and nontransformed cells. (*A* and *B*) MelHo (open symbols), IGR3 (▲), and A375 (●) are human melanoma cell lines (MelHo was not inhibited in adhesion; Table 1). (*C* and *D*) MRC-5 (○) are human lung fibroblasts, TuWi (●) is from human Wilm tumor, Cloudman melanoma (▲) is from mouse, and B 16-C 11 (△) is a highly metastatic subline of B 16 mouse melanoma (the adhesion of MRC-5 was not affected by the antibodies). NA, none absorbed.

Table 6. Reactivity of anti-B 16 monoclonal antibodies with *ts68*-RSV transformed CEFs

Antibody	Attached cells, % comparison with control	
	42°C	34°C
19/1	44	14
16/81	42	7
16/43	40	5
16/77	70	12
16/82	20	3

34°C, Permissive temperature; 42°C, nonpermissive temperature. Cells were cultured at 42°C and then shifted to 34°C for 10–14 hr. The adhesion assays were carried out at 37°C.

cells (a nonreacting human melanoma cell line; Table 1). The antibodies were not absorbed by either normal human fibroblasts (MRC-5; Fig. 2*C*) or mouse Cloudman melanoma cells (Fig. 2*D*; both were negative in the adhesion assay) but were bound by intact cells from Wilm tumor and B 16 mouse melanoma (Fig. 2*D*). Furthermore, the activity of selected antibodies was removed by living SV40-3T3 fibroblasts but not by untransformed 3T3 cells (Fig. 3*A*), which agrees with the adhesion data. Last, the mouse teratocarcinoma stem cells (F9, PCC4) express antigen 16/82 on their surface whereas the differentiated lines (PYS-2, 944) are negative (Fig. 3*B*).

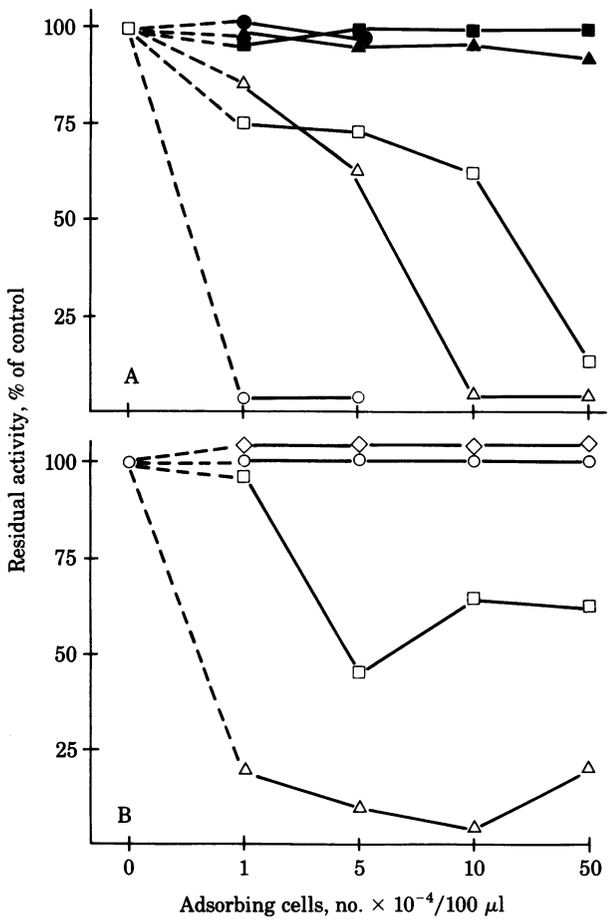

FIG. 3. Absorption of antibody activity by intact virally transformed fibroblasts and by teratocarcinoma cells. (*A*) Absorption by SV40-transformed 3T3 fibroblasts (open symbols); 3T3 fibroblasts served as a negative control (closed symbols). □ and ■, Antibody 16/43; ○ and ●, antibody 16/82; △ and ▲, antibody 16/51. (*B*) Absorption of antibody 16/82 by mouse teratocarcinoma cells. △, PCC4; □, F9 (nondifferentiated lines); ◇, PYS-2; ●, 944 (differentiated lines).

The molecular weights of the antigens recognized by selected anti-B 16 monoclonal antibodies were determined from immunoblots of $NaDodSO_4$ gels of whole cell lysates. When antibodies 16/43 and 16/82 were absorbed on blots from B 16 melanomas, both bound to antigens of M_r 40,000 (Fig. 4*A*) whereas antibody 19/1 was removed by an antigen having a M_r of 50,000 (Fig. 4*B*). Furthermore, blots from mouse 3T3 fibroblasts were unable to absorb these antibodies (Fig. 4*B*, shown for antibody 19/1). On blots of the human melanoma cells IGR3, antibody 19/1 recognized a similar antigen of M_r 50,000, whereas MelHo cells were negative to this antibody (Fig. 4*C*).

DISCUSSION

By three different procedures, we have determined that a series of monoclonal antibodies—which were obtained by syngeneic immunization against mouse melanoma cells and were functionally screened for inhibition of cell adhesion—crossreact with different human tumor cells: (*i*) they markedly interferred with adhesion of human tumor cells *in vitro*, (*ii*) they were quantitatively absorbed on intact human tumor cell surfaces,

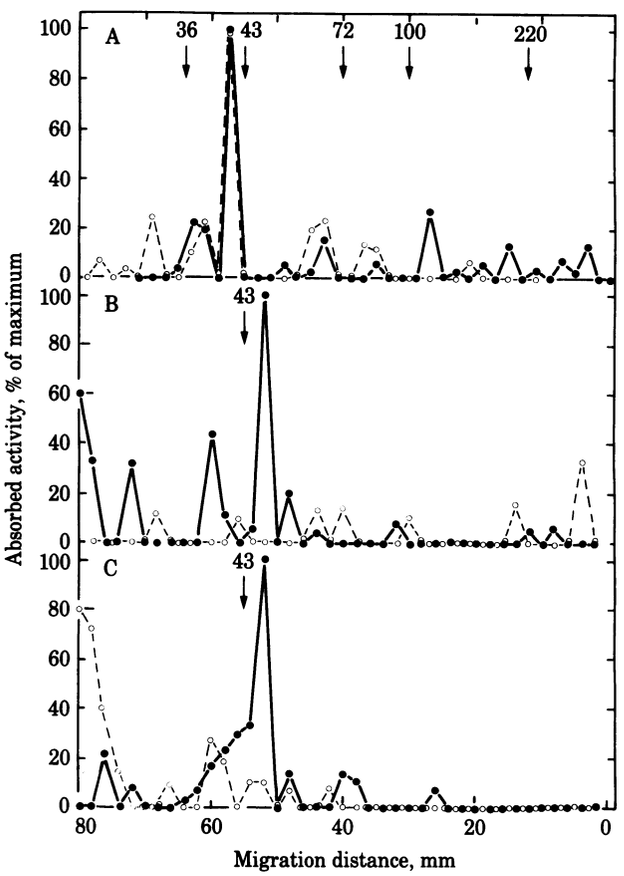

FIG. 4. Determination of molecular weights of the antigens. Immunological blots of $NaDodSO_4$ gels of whole cell lysates were analyzed for binding of antibodies 16/43, 16/82, and 19/1. The maximum of absorbance of the main peak in each experiment was taken as 100% after subtraction of the background. The critical antigenic sites are fully available after $NaDodSO_4$ gel electrophoresis, because 140,000 cell equivalents (cf. Fig. 2) resulted in maximum absorbance of activity. The molecular weights of the markers (36, 43, etc.) are given × 10^{-3}. (*A*) Antibodies 16/43 (●) and 16/82 (○) were analyzed on blots of B 16 mouse melanoma cells. (*B*) Antibody 19/1 was analyzed on blots of B 16 melanoma (●) and 3T3 mouse fibroblasts (○). (*C*) Antibody 19/1 was analyzed on blots of IGR3 (●) and MelHo (○) human melanoma cells.

and (*iii*) by immunological blotting of $NaDodSO_4$ gels, the corresponding antigens could be identified in human tumor cells. In all three assays, nontransformed cells were negative. Our data from this and the preceding work (12) suggest that preselection of syngeneically produced monoclonal antibodies against mouse tumor cells by suitable functional assays *in vitro* leads to reagents active against related functions in human tumor cell lines. It is significant that such antibodies interfere with animal tumor development.

The antigens that were detected here by syngeneic immunization seem to represent tumor-associated surface components. However, they were also found on embryonal teratocarcinoma cells although not on differentiated teratocarcinoma lines, and they were present in CEFs at early passages. It could thus be that they participate in some critical steps of embryonal adhesion and that tumor cells reexpress them. Three antibodies (16/43, 16/77, and 16/82) prevented the formation of experimental metastases *in vivo* (12), and the three antibodies so far analyzed biochemically (19/1, 16/43, and 16/82) recognize components with molecular weights of 40,000 or 50,000—i.e., at least two different antigens. However, their expression must be coupled. For instance, the antigens are present on SV40-transformed 3T3 cells, whereas they are absent from normal 3T3 fibroblasts. Apparently, the products of the transforming gene of SV40 virus (large and small tumor antigen; ref. 18) are capable of inducing the concomitant expression of a whole series of new surface antigens. The *src* gene product also seems to stimulate the expression of these tumor-associated surface antigens in chicken fibroblasts.

We have recently characterized another series of monoclonal antibodies, which prevent the *in vitro* adhesion of various cell types (19) or lead to the dissociation of epithelial cells (20). These antibodies were initially selected by functional and not, for example, radioligand binding assays; they often recognize minor cell surface antigens. Based on immunofluorescence studies, the anti-B 16 monoclonal antibodies are directed against even scarcer components on tumor cells (data not shown). Therefore, the molecular weights of the antigens were determined here by a novel procedure. We could absorb the functional activity of the antibodies on immunological blots of $NaDodSO_4$ gels loaded with whole cell lysates. This assay might be more widely applicable for the biochemical analysis of minor cellular components.

A series of human melanoma-associated antigens have recently been identified in other laboratories by means of both poly- and monoclonal antibodies (13, 21–30). We have not examined whether our syngeneically produced antibodies cross-react with any of these xenogeneically defined antigens. However, since the above-mentioned melanoma-associated antigens are all relatively major cell constituents (i.e., react strongly in conventional antibody binding assays) whereas our antigens are minor components and are present on a variety of different tumors, they may well not be identical.

A similar protein that is common to many tumors is cellular p53 (see ref. 31 for a recent review). This protein is expressed at high levels in transformed cells independently of the transforming agent and is not species specific. It is also detectable in nontransformed cells such as 3T3 fibroblasts but at concentrations lower by factors of 1/1,000 to 1/10. The tumor antigens detected by our syngeneically produced antibodies show a broad distribution similar to that of p53; they are present on melanomas, carcinomas, neuroblastomas, and teratocarcinomas; they are expressed on spontaneously (e.g., B 16), chemically (e.g., CSG 120/7 carcinoma), and virally (e.g., SV40-3T3 and RSV-CEF) transformed cells and they are not species specific. Our antigens are clearly distinct from p53, however, because they all seem to be surface adhesion molecules.

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